

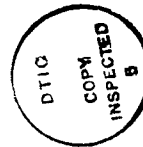
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## NEW APPROACHES TO HEPATITIS A VACCINE DEVELOPMENT

ANNUAL REPORT

STANLEY M. LEMON

APRIL 22, 1991



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## FOREWORD

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The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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## ABSTRACT

Hepatitis A virus (HAV) has historically been an important cause of morbidity among U.S. soldiers in the field. Work under this contract is directed at the development of a safe, inexpensive and effective hepatitis A vaccine for use in military personnel. As in the preceding year, two main research approaches are under investigation. First, the primary goals of this contract are the characterization of neutralization epitopes on the surface of the HAV capsid and an examination of the antigenicity of synthetic oligopeptides representing the suspected surface structures of HAV. Octapeptides have been synthesized on polyethelene pins and probed with polyclonal and monoclonal antibodies in an effort to identify antigenic and potentially immunogenic sequences. We also continue the investigation of poliovirus-HAV chimeric viruses which have been engineered to express potential HAV neutralization determinants, and in addition have examined the effects of specific proteases on the antigenic determinants and capsid proteins of the HAV virion. The second major approach involves basic studies related to genetic mechanisms of attenuation of HAV. This work has involved construction of a genomic length cDNA construct derived from a virulent but cell culture-adapted variant of HAV, and studies aimed at characterizing the secondary structure and function of the 5' nontranslated region (5' NTR) of HAV. The 5' NTR plays a key role in determining the attenuation phenotype of the Sabin poliovirus vaccine strains, and these studies may lead to new approaches to the construction of genetically engineered attenuated vaccine candidates. A third aim has been the characterization of genetic diversity among wild-type HAV isolates using PCR-related technology, as this will facilitate clinical studies of HAV vaccines and future military disease surveillance activities.

## INTRODUCTION

The recent large scale mobilization of the military forces of the United States during Operations Desert Shield and Desert Storm have reemphasized the risks and special problems posed by hepatitis A virus (HAV) to American military operations. Due to substantial improvements in public health sanitation within the United States over the past five decades, the prevalence of HAV has continued an overall decline within most regions of the country. As a result, American forces personnel demonstrate an extremely low prevalence of antibodies to the virus (Lemon and Bancroft, 1983). Compared with the military forces of many other nations, American soldiers, marines and sailors have a relatively low prevalence of previously acquired immunity to HAV and are thus particularly susceptible to this potentially debilitating infectious disease. Among American civilian populations, overseas travel to HAV endemic regions represents a substantial risk factor for acquisition of hepatitis A. This risk is substantially magnified when Americans traveling overseas are military forces deployed to developing regions. Even greater risks may be anticipated when previously existing public health facilities and sanitation practices have been disrupted by military conflict, such as occurred within the Kuwaiti Theater of Operations. While short-term protection against hepatitis A may be provided by passive administration of immune globulin (IG), supplies of IG may be strained during massive mobilizations such as Desert Shield. Moreover, readministration of IG is required at 6 months intervals in order to maintain continued protection and this may be particularly difficult to accomplish with troops engaged in action against hostile forces. There is thus an urgent need for development of a vaccine capable of providing safe, long-term, active immunity against HAV, and which would be available to the military forces of the United States at reasonable cost.

Commercial interest in development of formalin-inactivated HAV vaccines has resulted in products that appear safe and reasonably immunogenic in phase I and early phase II clinical trials (for a recent review, see Siegl and Lemon, 1990). However, there are several unresolved questions concerning the use of such vaccines. The magnitude and speed of the neutralizing antibody response to inactivated HAV appears directly related to the quantity of inactivated viral antigen included in each dose. Vaccines containing less than 200-400 ng purified antigen appear to require multiple administrations in order to elicit protective levels of immunity. Multiple-dose schedules, with late booster doses given at 6 months in several current clinical trials, may prove inconvenient for use in military populations. Perhaps of greater practical significance, the future cost of such vaccines is unknown but likely to be quite high. This is due to the comparatively poor in vitro yields of antigen obtained with current vaccine virus strains, and the purification procedures required for production of an acceptable, modern vaccine. High costs will probably prohibit the universal use of inactivated vaccines among U.S. military forces. Because of the uncertainties concerning future use of inactivated HAV vaccines, work under this grant focuses on efforts to develop alternative approaches to development of HAV vaccines.

In previous work, we have mapped an immunodominant neutralization antigenic site on the surface of the HAV capsid by analysis of murine monoclonal antibody-resistant neutralization escape variants of HAV (Ping et al., 1988; Cox et al., 1990), and by characterizing the competition between

such monoclonal antibodies for binding to the virus capsid (Stapleton et al., 1987). These studies indicate that the AB-AC loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988; Day et al., 1990a). Although, this site is largely conformationally defined, we have reasoned that short oligopeptide sequences representative of the relevant regions of VP3 and VP1 should be both antigenic and potentially immunogenic with respect to HAV neutralizing activity. This has been shown to be the case with peptides representative of antigenic sites in two other picornaviruses that are closely related to HAV, type 1 poliovirus (Chow et al., 1985) and human rhinovirus 14 (Francis et al., 1989), as well as the more unique foot-and-mouth virus (FMDV) (Bittle et al., 1982). Peptide immunogens are highly stable reagents; they are potentially very inexpensive and extremely safe inasmuch as they are chemically defined. As we have previously shown that only low levels of neutralizing antibody are required for protection against hepatitis A (Stapleton et al., 1985), we have postulated that peptide immunogens may have practical application to the prevention of hepatitis A.

We have taken several experimental approaches to this problem. First, we have continued efforts to map the neutralization epitopes of HAV by the isolation and characterization of monoclonal antibody resistant neutralization escape HAV mutants. During the past year, we have developed significant new information concerning the antigenic structure of the HAV capsid utilizing this approach. A second approach has involved the synthesis of octapeptides representing the primary sequence of the HAV capsid proteins VP3 and VP1 on polyethylene pins, and the probing of such peptides with monoclonal and polyclonal anti-HAV antibodies in peptide ELISAs (PEPSCAN) (Geysen et al., 1984, 1987). A third approach which we have pursued in collaboration with Prof. J. Almond of the University of Reading, Reading, Berks., U.K. and Dr. P. Minor of the National Institute of Biological Standardization and Control, Potters Bar, Herts., U.K., has been the insertion of appropriate HAV peptide sequences into an antigenic loop of capsid protein VP1 of the Sabin type 1 virus. These chimeric picornaviruses have been constructed using an infectious poliovirus cDNA clone which contains a mutagenesis cassette in the region encoding for VP1 (Burke et al., 1989). HAV/poliovirus chimeras have the potential of presenting HAV peptides in a conformationally constrained manner, and we have been encouraged by preliminary success with such chimeric viruses (see Progress Report No. 1).

In addition to exploring these approaches to subunit HAV vaccines, we have continued efforts to develop an infectious HAV cDNA clone derived from a virulent but highly cell culture-adapted virus (p16 HM175). This work follows on from that supported under a previous contract with the U.S. Army Medical Research and Development Command (DAMD17-85-C-5272). Commercial attempts to develop an attenuated HAV vaccine have focused on the attenuation of HAV that follows adaptation of virus to cell culture (for a review, see Lemon, 1985; Siegl and Lemon, 1990). Such viruses appear to replicate poorly in the primate liver, and have very poor immunogenicity in man. There is thus a need for novel approaches to selecting attenuated HAV vaccine candidate strains.

Development of an infectious, virulent construct would be useful in further characterizing the molecular basis of adaptation of virus to growth in cell culture, as well as attenuation of HAV. A genomic-length cDNA clone derived from p16 HM175 (Jansen et al., 1988) has been constructed within the

transcriptional vector pGEM-3. This construct was not viable in transfection assays, either as RNA or DNA, but transfection experiments with chimeric molecules constructed with a viable, but attenuated full-length clone (pHAV/7, Cohen et al., 1987) suggested that a lethal mutation existed between bases 4977 and 7003 of the pl6 clone (see Progress Report No. 1). Complete sequencing of the pl6 construct demonstrated a lethal, frame shifting mutation within this region. This defect has been corrected and the clone is now being examined for infectivity in transfection experiments.

In related work during the past year, we have characterized the secondary structure and translational control functions of the 5' nontranslated region (5' NTR) of the HAV genome. This work has provided valuable new information concerning the functional organization of this genetic element. In the Report from the preceding year, we suggested that deletions within the 5' NTR such as those made in virulent, Mahoney type 1 poliovirus by Iizuka et al. (1989) might lead to attenuation. We now know that the HAV 5' NTR structure and organization differs fundamentally from that of poliovirus and that this approach is unlikely to work. However, we have shown that the HAV 5' NTR structure is very similar to that of the murine cardioviruses, leading us to suspect that measures taken to attenuate cardioviruses by creating deletion mutations within the cardioviral 5' NTR may be highly relevant to the genetic engineering of an attenuated HAV vaccine.

Finally, we reported last year the development of a simplified polymerase chain reaction (PCR)-based method for analysis of the nucleotide sequence of wild-type HAV isolates. We helped to establish this antigen-capture/polymerase chain reaction (AC/PCR) method (Jansen et al., 1990) within the Department of Virus Diseases of the Walter Reed Army Institute of Research, where it is currently being used in support of ongoing clinical trials of HAV vaccines, and is undergoing modifications for the detection of hepatitis E virus. During the past year, we have also collaborated with the Viral Hepatitis Branch of the Centers for Disease Control in an effort to evaluate the genotype of a large number of HAV isolates. This effort has resulted in the analysis of virtually all available HAV strains, and has provided new information concerning the genetic diversity of HAV and the molecular epidemiology of the virus.

## RESEARCH PROGRESS

### 1. Neutralization epitopes of HAV

In previous work, we mapped an immunodominant neutralization antigenic site on the surface of the HAV capsid by analysis of murine monoclonal antibody-resistant neutralization escape variants of HAV (Ping et al., 1988; Cox et al., 1990), and by characterizing the competition between such monoclonal antibodies for binding to the virus capsid (Stapleton et al., 1987). These studies indicated that the B-C loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988; Day et al., 1990). Virus mutants having substitutions at the Asp residue 70 of VP3 were shown to be resistant to the majority of available murine monoclonal antibodies, while those with substitutions within the B-C loop of VP1 had partial resistance to



a much smaller number of monoclonal antibodies. Surprisingly, cross resistance between these mutants suggested that both VP3 and VP1 domains contributed to the same antigenic site (Ping et al., 1988).

We have extended these studies by the isolation of additional neutralization escape variants of HAV. Normally a very difficult task given the noncytopathic slow growth and relatively poor neutralization of HAV in vitro, this effort was enhanced by the use of a rapidly replicating, cytopathic virus variant (HM175/18f) (see Section 9 below) (Lemon et al., 1991) and development of a double-antibody radioimmunofocus assay (Lemon et al., 1990) capable of distinguishing individual replication foci of antigenically variant viruses. New HAV escape mutants were isolated using a protocol involving the initial neutralization of a relatively large virus pool ( $>10^6$  RFU) with a high concentration of murine antibodies. Virus-antibody mixtures were washed off cell sheets after a relatively short adsorption period, in an effort to reduce the nonspecific virus surviving fraction. The cells were refed with media containing lesser concentrations of antibody (one tenth that used for neutralization). Surviving virus was subjected to repeated cycles of neutralization and passage in the presence of antibodies at weekly intervals, with virus harvests tested for neutralization resistance by double-antibody radioimmunofocus reduction assay after the third and sixth cycle. Escape mutants were twice plaque-purified from radioimmunofocus assay cell cultures, and amplified in 25 cm<sup>2</sup> flasks of BS-C-1 cells. AC/PCR (Jansen et al., 1990) was utilized for partial sequencing of the viral genome within the P1 capsid protein encoding region.

Several murine monoclonal antibodies were selected for these studies, based on available quantities and the results of previous studies. Multiple mutants were selected from each (see Figure 1). We had previously demonstrated that VP3 residue 70 (by convention, 3-070<sup>1</sup>) mutants resisted neutralization by the monoclonal 2D2. In order to more completely map this antigenic site, and to determine whether this antibody would select for mutations within the B-C loop of VP1 as well as VP3, we isolated 6 independent 2D2 escape mutants. The mutations within all of these mutants have been mapped to VP3 (residues 3-070 and 3-074) (Figure 1). Mutants were selected against AD2, AE8, and H7C27 as these antibodies continued to neutralize previously isolated 3-070 mutants, and thus might recognize unique sites on the HAV capsid. Mutations within these escape mutants localized to residues 1-171 and 1-176 (AD2 and AE8), and 2-221 (H7C27), thus indicating the involvement of two additional capsid protein loops in the neutralization antigenic domains of HAV. Multiple, independently isolated mutants had identical capsid mutations (Figure 1). These results suggest that the murine antibodies have high level specificities for selection of certain mutations, and that the original viral inoculum may contain a relatively limited number of viral pseudospecies having mutations in the capsid proteins. This latter interpretation is not surprising given the overall genetic stability of the P1 region during passage in cell culture (Jansen et al., 1988; Lemon et al., 1991).

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<sup>1</sup>By convention, HAV capsid protein amino acid residues are designated by four-digit numbers, the first of which indicates the specific capsid protein, followed by three digits indicating residue number.

Escape mutants with unique capsid mutations listed in Figure 1 were studied for resistance to neutralization by the complete panel of monoclonal antibodies (see Figure 2). While yet incomplete, these very labor intensive studies indicate that mutants with escape mutations at 3-070, 3-074, 1-102, 1-171, and 1-176 all share cross-resistance to certain common monoclonal antibodies, although resistance to the 1-171 and 1-176 mutants is found only with a small subset of antibodies. These data suggest that all of these residues contribute to a single antigenic site composed of a variety of functionally overlapping epitopes. In contrast, all data collected so far indicate that the 1-221 mutants are sensitive to neutralization by other monoclonal antibodies and that this residue thus contributes to a second, functionally independent site. We consider this second antigenic site to be immunorecessive for two reasons: (1) only one of 20 monoclonal antibodies reacts with this site, and (2) preliminary studies suggest that the 1-221 mutants (unlike the 3-070 mutants) have normal antigenicity when tested for the ability to be recognized by polyclonal antibodies. Significantly, the cross-neutralization studies indicate that two monoclonal antibodies (H29C26 and 1.193) continue to neutralize all available mutants, which suggests that other potentially independent neutralization sites might exist on the capsid surface.

We have analyzed the position within the crystallographically defined structure of mengovirus of amino acid residues which align with the sites of HAV escape mutations (Figure 3). The capsid protein alignments of Palmenberg (1989) were used for this purpose. Significantly, these sites are all found on surface exposed loops of mengovirus VP3 and VP1, indicating significant similarities in the structures of these viruses. The location of the 1-221 mutation is of particular interest as it occurs within the "foot-and-mouth disease virus (FMDV) loop" of mengovirus. In FMDV, this loop contains a linear neutralization epitope which functions well as a peptide immunogen (Bittle et al., 1982). In order to evaluate the potential for a linear site within this loop region of HAV, we will carry out pin-based oligopeptide synthesis of the carboxy half of VP1 (residues 1-131 to 1-300) during the next year of this grant.

## 2. PEPSCAN analysis of HAV capsid proteins

The specific approach we have taken to mapping antigenic peptides of HAV involves the synthesis of nested octapeptides on polyethylene pins. These peptides, overlapping each other by 7 residues, are probed in enzyme-linked immunosorbent assays (ELISA) which assess the binding of immunoglobulins to specific peptide-bearing pins (PEPSCAN) (Geysen et al., 1984, 1987) (see Report No. 1 for this grant submitted in 1990). During the past year we screened pin-based octapeptides representative of the putative B-C loops of VP3 (residues 3-050 - 3-091) and VP1 (residues 1-080 - 1-130) of HAV against a panel of 24 neutralizing anti-HAV monoclonal antibodies. No reactivity was demonstrated with these monoclonal antibodies, even though analysis of escape mutants (see above) indicates that some of these antibodies recognize epitopes which are comprised, in part, of amino acid residues 3-070, 3-074 or 1-102. Thus, these epitopes are highly conformational and do not contain linear, antigenic peptides. As indicated above, we are extending the PEPSCAN analysis to include the region from 1-131 to 1-300, as we have shown that this region contains two additional domains involved in epitopes recognized by neutralizing monoclonal antibodies.

### 3. Analysis of HAV/poliovirus chimeras

During the previous year we initiated a collaboration with Prof. J. Almond of the University of Reading, Reading, Berks., U.K. and Drs. P. Minor and M. Ferguson of the National Institute of Biological Standardization and Control (NIBSC), Potters Bar, Herts., U.K. in order to evaluate chimeric picornaviruses in which potential HAV epitopes have replaced part of the antigenic VP1 B-C loop of Sabin type 1 poliovirus. The availability of an infectious cDNA clone of the Sabin type 1 virus containing a mutagenesis cassette in the region encoding the VP1 B-C loop (Burke et al., 1989) has facilitated the construction of these HAV/poliovirus chimeras. To date a total of 17 viable poliovirus/HAV chimeras expressing a variety of HAV sequences have been generated (see Table 1). Antisera have been raised against these viruses in small animals at NIBSC. We have carried out HAV neutralization tests with anti-chimera antisera and assessed the HAV antigenicity of chimeric viruses.

Table 1. HAV Neutralizing Activity of PV1/HAV Chimera Antisera

Chimera	HAV residues	Positive* antisera/Number tested		
		Rabbit	Guinea Pig	Mouse
H15	VP1 13-24	3/9	0/4	1/1**
H1	VP1 15-20	nd	0/3	0/3
H11	VP1 29-41	nd	0/2	0/3
H14	VP1 70-81	nd	0/1	nd
H10	VP1 99-107	0/5	0/6	0/3
H20	VP1 99-122	0/2	nd	nd
H2	VP1 101-108	1/4	0/8	0/3
H19	VP1 111-123	0/2	nd	nd
H3	VP1 150-155	nd	0/3	0/3
H18	VP1 166-178	0/2	nd	nd
H5	VP1 192-197	nd	0/3	0/3
H6	VP1 217-222	nd	0/2	0/3
H12	VP1 289-300	nd	nd	nd
H13	VP2 49-55	nd	0/1	nd
H17	VP3 63-74	nd	nd	nd
H16	VP3 68-79	0/5	0/4	nd

\* >80% neutralization at 1:5-1:10 dilution

\*\* pool of 3 mouse sera

The results of HAV neutralization assays with anti-chimera antisera are summarized in Table 1. These represent an extension of the data reported last year. Neutralization of HAV was assessed in radioimmunofocus-reduction assays. At serum dilutions of 1:10, significant neutralizing activity was present in 3 of 9 immunized rabbits and in a single mouse serum pool raised to the HAV/poliovirus chimera S1/H15, while 1 of 4 rabbits developed neutralizing antibodies following immunization with chimera S1/H2. Positive results were reproducible, and rabbit prebleeds were devoid of neutralizing activity in each case. The mouse anti-S1/H15 pool had the highest anti-HAV titer

(approximately 1:100). Although several anti-S1/H15 sera neutralized HAV, none were capable of immunoprecipitating <sup>3</sup>H-uridine labelled HAV. To confirm that the low levels of neutralizing activity elicited by S1/H15 and S1/H2 chimeras represented specific neutralizing antibody, we purified IgG antibody from serum samples by FPLC and tested isolated immunoglobulin for neutralization activity. These results (data not shown) confirmed that the neutralization is antibody-mediated, as purified IgG neutralized virus effectively, while depletion of IgG from a positive rabbit serum removed neutralization activity.

The S1/H15 chimera contains residues representing an amino terminal domain of HAV VP1. A synthetic peptide with similar sequence was reported previously by Emini et al. (1985) to be immunogenic and capable of eliciting neutralizing anti-HAV activity. An analogous poliovirus peptide has similarly been reported to induce neutralizing antibody to poliovirus by Chow et al. (1985), despite the fact that this VP1 domain has an internal position within the native poliovirus capsid (Hogle et al, 1985). However, Frick and Hogle (1990) have recently demonstrated that the amino terminus of VP1 of poliovirus is externalized during the conformational shift to 135 S particles ("A" particles) that accompanies cell attachment and early steps of penetration of poliovirus. This sequence of events provides a potential explanation for the neutralizing activity of antisera directed against the VP1 amino terminus of HAV, and may explain why anti-S1/H15 antisera which neutralize HAV infectivity fail to immunoprecipitate native virus.

To better understand the mechanism by which anti-S1/H15 antibody mediated neutralization, we carried out neutralization at 4° C. Virus-antibody mixtures were allowed to adsorb to BS-C-1 cells at this temperature for a period of 30 min, then washed at 4° C before addition of the radioimmunofocus overlay. Under these conditions, each of three rabbit anti-S1/H15 sera tested effected a ≥80% reduction in HAV radioimmunofoci. With each sera, neutralization activity appeared somewhat enhanced at 4° C compared with identical neutralizations carried out 37° C (data not shown). These results indicate that the putative conformational change which results in the exposure of the HAV N-terminal neutralization domain occurs with attachment of virus at 4° C, and does not require energy-dependent cellular penetration. Neutralization may be enhanced at the lower temperature because virus penetration is blocked and conformationally altered virus thus remains exposed to potential antibody binding at the cell surface.

We are uncertain why only one-third of rabbits immunized with S1/H15 develop neutralizing antibody activity (Table 2). One potential explanation may be that the proportion of denatured virus differed in the inocula used for each animal series and that denatured virus might be more immunogenic (results in Table 2 represent the results of four different series of immunizations). To test this hypothesis, we immunized two rabbits (R118 and R119) with virus held at 56° C for 10 minutes, conditions which convert 155S virions to 70S empty capsids. As shown in Table 2, no neutralizing response occurred in these two rabbits, although one of two control rabbits simultaneously immunized with non-heat denatured virus developed a 1:10 neutralization titer. Thus denatured virus does not appear significantly more immunogenic than native virus. The relatively poor neutralizing response in immunized animals, and the inconsistent response noted with similar peptide immunogens may reflect conformational attributes of this accessory neutralization site.

Alternatively, the peptide sequences examined thus far may only partially represent this site. Over 20 murine monoclonal antibodies specific for the S1/H15 HAV peptide insert were raised by immunization of mice with S1/H15 in collaboration with M. Ferguson of NIBSC. None of these monoclonal antibodies neutralized HAV.

Table 2. Anti-viral and Anti-peptide Activity of S1/H15 Antisera

Antiserum	S1/H15 doses	Anti-H15 (RIP)	Anti-Peptide <sup>1</sup> (ELISA)	Anti-HAV (neutralization)
M18 pool	4	3.3 <sup>2</sup>	2.0 <sup>2</sup>	2.0 <sup>2</sup>
R81 1.08	4	2.6	1.0	1.0
R82 1.08	4	2.8	1.0	1.0
R93 15.12	3	3.8	3.0	<1.0
R94 15.12	3	4.0	3.0	<1.0
R100 10.01	3	4.3	4.0	<1.0
R101 10.01	3	4.4	3.0	<1.0
R102 10.01	3	3.9	4.0	<1.0
R116 18.09	3	nd	nd	<1.0
R117 18.09	3	nd	nd	1.0
R118 18.09 <sup>3</sup>	3	nd	nd	<1.0
R119 18.09 <sup>3</sup>	3	nd	nd	<1.0

<sup>1</sup>Peptide 639 (1011-1032)

<sup>2</sup>Log<sub>10</sub> serum titer

<sup>3</sup>Immunized with heat-denatured virus (56° C for 10 min)

One of 4 rabbits immunized with the S1/H2 chimera (HAV residues 1101-1108) developed significant anti-HAV neutralizing antibodies. The HAV domain included in the chimeric virus in this case includes a residue known to be the site of a neutralization escape mutation (residue 1102), and the chimera most likely represents a VP1 B-C loop replacement (which results in immunogenic inter-typic poliovirus chimeras). However, although seroconversion in a single animal is encouraging, other rabbit, guinea pig and mouse sera were negative for HAV neutralizing activity (Table 1). We constructed additional chimeras in which the inserted HAV sequence was extended to include residue 1114 of HAV. However, antisera raised to these chimeras (S1/H19 and S1/H20) failed to neutralize HAV (Table 1). This was also the case with antisera to S1/H18 and S1/H6 (Table 1), which express HAV peptide sequences containing the sites of recently identified escape mutations (Figure 1).

#### 4. Effects of proteolytic enzymes on HAV capsid proteins and antigenicity

In an effort to better characterize the antigenic structure of HAV, we examined the effect of sequence-specific proteolytic enzymes on the capsid proteins, antigenicity and infectivity of the virus. High concentrations of either trypsin or chymotrypsin (10 mg/ml) resulted in cleavage of VP2 that was evident in immunoblots with anti-peptide antibodies specific for each of the three major capsid proteins. Chymotrypsin also had a reproducible but lesser effect on VP1. Despite nearly complete cleavage of VP2, there was no reduction in infectivity or in thermostability of the virus between 50 °C and

60 °C. Cleavage of VP2 and VP1 also had no demonstrable effect on viral antigenicity, including the ability of virus to bind to and be neutralized by murine monoclonal antibodies. No cleavage of viral proteins was observed following digestion with staphylococcal V8 protease (5 mg/ml), or lesser concentrations of endoproteinase Lys-C or endoproteinase Arg-C. These findings thus indicate the presence of a protease-accessible VP2 surface site which neither contributes significantly to the immunodominant antigenic site nor plays a role in attachment of virus to putative cell receptors.

#### 5. Construction of infectious cDNA of a virulent HAV variant

We have continued efforts to develop an infectious cDNA clone derived from a virulent but highly cell culture-adapted HAV variant (HM175/P16) (Jansen et al., 1988). Such a construct would be useful in further characterizing the molecular basis of adaptation of virus to growth in cell culture, as well as attenuation of HAV. A genomic-length cDNA clone derived from HM175/P16 (pHAV16) has been assembled within the transcriptional vector pGEM-3. Details of this construction may be found in the final report of our previous contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272), and in Report No. 1 of this grant which we submitted last year. The pHAV16 construct unfortunately has not yielded infectious virus following transfection of BS-C-1 cells with either RNA or plasmid DNA. To identify the lethal mutation(s) within the clone, we constructed a series of genomic-length chimeric cDNAs containing variable contributions from the pHAV16 and the infectious pHAV/7 clones. Transfection of BS-C-1 cells with these chimeric plasmids suggested the presence of a lethal mutation between HAV map positions 4977 and 7003 of the pHAV16 construct (see Report No. 1 submitted last year). Resequencing of the entire pHAV16 construct revealed a frame-shifting mutation at base 6895. This region has been replaced with cDNA from an alternative cDNA clone, and the revised construct is currently being tested for viability. This is being accomplished by lipofectin-mediated transfection of BS-C-1 cells with RNA synthesized under direction of SP6 polymerase. We are confident that an infectious cDNA clone of a virulent but cell culture adapted HAV variant will be available within the very near future.

#### 6. Role of 5' NTR mutations in adaptation of HAV to growth in cell culture

Previous comparisons of mutations present in cell culture-adapted HM175 virus variants have suggested that mutations within the 5' nontranslated region (5' NTR) may play an important role in altering the host range of the virus and in determining attenuation (Jansen et al., 1988). To assess the impact of these mutations on the replication competence of cell culture-adapted HAV in cell culture, we constructed several chimeric viruses in which regions of the 5' NTR of pHAV/7 have been replaced with sequences derived from wt or the cell culture-adapted but virulent HM175/P16 virus (Jansen et al., 1988). The capacity for growth of these viruses in BS-C-1 cells has been assessed by determining focus-size ("plaque" size) in radioimmunofocus assays. These studies represent an extension of those described in last years report. SP6 transcripts made from plasmid DNA containing chimeric 5' NTR sequences were transfected into BS-C-1 cells using a liposome-mediated transfection strategy, and virus harvests were taken at two weeks. The replication competence of the rescued chimeric viruses was assessed by subsequent determination of the replication focus-size in radioimmunofocus assays (RIFA) (Lemon et al., 1983) (essentially "plaque" size).

Our results (Figure 5) indicate the following contributions of 5' NTR mutations to growth in cell culture. First, substitution of the complete 5' NTR of HM175/P35 with the 5' NTR of HM175/P16 (pHAV/7-5'P16) resulted in viable virus with RIFA focus size comparable to that of HM175/P35. On the other hand, replacement of the 5' NTR of HM175/P35 with the 5' NTR of wild-type virus (pHAV/7-5'wt) resulted in a viable chimeric virus with a very small focus phenotype resembling that seen with wild-type virus in cell culture (Jansen et al., 1988). Thus, mutations within the 5' NTR are important for growth in cell culture. The fact that the 5' NTRs of HAV/7 and HM175/P16 can freely substitute for each other suggests that the mutations which P16 and P35 viruses share in common within this region (bases 152 and 203-7) may be particularly important to adaptation to growth in cell culture. We tested this hypothesis by constructing two additional plasmids in which HM175/P16 mutations were excluded at base 687 (pHAV/7-5'P16(0-528)) and also at bases 0 and 8 (pHAV/7-5'P16(24-528)). Virus rescued from these plasmids generated replication foci of intermediate size, indicating a definite but relatively minor contribution from the mutation at 687 (Day et al., 1990). These data thus indicate that the mutations at bases 152 and/or 203-7 are of critical importance in determining the ability of virus to replicate in BS-C-1 cells.

Base 687 lies within the putative internal ribosomal entry site (IRES) of the HAV 5' NTR (see below). We thus examined the translational efficiency of different HAV 5' NTR constructs (wt, HM175/P16 and HM175/P35) in vitro in rabbit reticulocyte lysates (data not shown). We found no differences in the translational efficiencies of full length RNA transcripts made from these constructs. However, these data do not rule out a role for these mutations in enhancing translation in vivo. We suspect that these mutations (at least the mutation at base 687) may promote the interaction of viral RNA with specific cellular proteins involved in translation initiation. In fact, if the 5' NTR mutations do represent an adaptation from growth in hepatocytes in vivo to growth in monkey kidney cell cultures, it would have been surprising were we able to demonstrate a specific functional difference in lysates of nonprimate reticulocytes.

## 7. A model of the secondary structure of the 5' NTR of HAV

Phylogenetic analysis of sequence covariance has provided useful information concerning the secondary structure of the 5' NTRs of enteroviruses (Rivera et al., 1988; Pilipenko et al., 1989; Skinner et al. 1989), cardioviruses and aphthoviruses (Pilipenko et al., 1989b). However, it has not been possible to apply this approach to HAV because the sequence of the 5' NTR of HAV differs from that of other picornaviral 5' NTRs to such an extent that meaningful alignments are not possible, and because the degree of conservation within the published 5' NTR sequences of human HAV strains is insufficient for successful analysis of sequence covariance. This situation changed, however, with our identification of genetically divergent simian and human strains of HAV (Brown et al., 1989; Jansen et al., 1990).

To initiate a phylogenetic analysis of the 5' NTR of HAV, we determined the nearly complete sequences of the 5' NTRs from two genetically divergent HAV strains (PA21 and CF53) and aligned these sequences with 5 published human HAV 5' NTR sequences, and the 5' NTR sequences of KRM003 (provided to us by Y. Moritsugo and A. Nomoto) and AGM27 (provided to us by S. Tsarev, S. Emerson and R. Purcell). We manually identified covariant nucleotide substitutions

predictive of conserved secondary structures, and utilized this information to develop a model of the 5' NTR secondary structure which was further refined by thermodynamic predictions using RNAFOLD (University of Wisconsin Genetics Computer Group Software Package) and STAR (Abrahams et al., 1990) programs. The model structure was subsequently tested and further refined by nuclease digestion experiments in which we determined the effects of single- and double-strand specific ribonucleases on synthetic RNA (SP6 transcripts from a plasmid construct containing HM175/P16 HAV cDNA).

The resulting model suggests that the HAV 5' NTR comprises six major structural domains (Figure 6). Domains I and II (bases 1-95) contain a 5' terminal hairpin and two stem-loop structures that are capable of forming pseudoknots. This set of structures is quite different from that present at the 5' terminus of the poliovirus genome, but very similar to that predicted for EMCV (Pilipenko et al., 1989b). This region is highly conserved among HAV strains studied thus far, resulting in the absence of identifiable covariant substitutions. The validity of the predicted structure is enhanced by the fact that it is comprised entirely of canonical A-U, G-C base pairs (excluding noncanonical pairs such as G-U), and the presence of considerable structural homology with the cardio- and aphthovirus 5' termini (Pleij et al., International Congress of Virology, Berlin, August, 1990). In HAV, this region is followed by a highly variable polypyrimidine tract (poly-Y<sub>1</sub>, bases 96-154), which is very likely to be single-stranded because it is subject to extreme variability without apparent sequence covariance. The remainder of the 5' NTR (domains III-VI, bases 155-734) contains several complex stem-loops, one of which may form a pseudoknot, and terminates in a highly conserved region containing an oligopyrimidine tract (poly-Y<sub>2</sub>) preceding the putative start codon by 13 bases. The 3' half of this region is well defined by numerous covariant base substitutions among different HAV strains, while further sequencing of HAV strains must be accomplished in order to better establish the secondary structure of the 5' domain. It is important to note that the extreme 3' stem-loop/poly-Y<sub>2</sub> domain has striking structural similarities to the 3' region of the IRES element proposed for EMCV.

#### 8. Structural elements required for internal ribosomal entry in HAV

Although translation of host cell, capped mRNAs is not abrogated in HAV-infected cells, the fact that translation of the HAV polyprotein is initiated at the 11th or 12th AUG codon from the 5' end of the viral RNA suggests that initiation of HAV translation follows internal binding of the 40S ribosomal particle within the 5' NTR, as has been suggested for other picornaviruses. To determine which 5' NTR structural elements might contribute to an IRES element in HAV, we constructed a series of plasmids containing progressive 5' deletions within the HAV 5'NTR. The parent construct from which these plasmids were made was pHAV/P16, which contains the complete genomic sequence of the cell culture adapted HM175/P16 strain downstream of the SP6 promoter. RNA transcripts representing the HAV 5' NTR with progressive 5' deletions, followed by a truncated P1 region, were translated in vitro in rabbit reticulocyte lysates (Promega). The translation products were immunoprecipitated with anti-peptide antibodies and analyzed by SDS-PAGE. Approximately 200 ug of synthetic RNA was included in each translation reaction, so that RNA quantity was not limiting.



These experiments demonstrated that removal of the the 5' terminal hairpin had only a slight, but definite, negative effect on translation efficiency (Figure 7). This may reflect some role for the 5' hairpin in translation of HAV proteins, as Simoe and Sarnow (1991) recently suggested for the analogous structure in poliovirus, or a reduction in RNA stability. Further deletion up to base 354 of the 5' NTR had no effect on translation. However, deletion to base 447 further decreased translation, while deletion to 533 almost completely abolished it. Removal of the 5' 63 bases restored translation to a level greater than that achieved with the full length 5' NTR. This finding likely represents efficient initiation of translation by conventional 5' scanning of the 5' truncated, uncapped RNA (a phenomenon known to occur in reticulocyte lysates), and indirectly suggests that the 40S ribosomal subunit has great difficulty in translocating across the region from base 533 to 634.

The results of these in vitro experiments indicate that the 5' NTR sequence 3' of base 354 plays an important role in the translation mechanism utilized by genomic-length HAV RNA. The data suggest that the HAV 5' NTR contains an IRES element located within this domain and that it thus shares an overall functional organization similar to that of other picornaviruses. This is consistent with the structural similarities evident between the 5' NTRs of HAV and the cardio- and aphthoviruses.

#### 9. Mutations associated with cytopathic HAV

We reported in the preceding annual report that HAV variants recovered from persistently infected green monkey kidney (BS-C-1) cells (pHM175 virus) induced a cytopathic effect during serial passage in BS-C-1 or fetal rhesus kidney (FRhK-4) cells. Epitope-specific radioimmunofocus assays showed that this virus comprised two virion populations, one with altered antigenicity including neutralization-resistance to monoclonal antibody K24F2, and the other with normal antigenic characteristics (Lemon et al., 1991). We demonstrated that replication of the antigenic variant was favored over virus with normal antigenic phenotype during persistent infection, while virus with the normal antigenic phenotype was selected during serial passage. Viruses of each type were clonally isolated; both were cytopathic in cell cultures and displayed a rapid replication phenotype when compared to the noncytopathic HM175/P16 virus which was used to establish the original persistent infection. The clonal variant which has normal antigenic phenotype (HM175/18f) has exceptional in vitro growth properties, and has been supplied to Dr. Leonard Binn of the Department of Virology, Walter Reed Army Institute of Research for use in HAV neutralization assays done in support of HAV vaccine trials sponsored by WRAIR.

We determined the nearly complete genomic sequences of the two cytopathic virus clones HM175/43c (the antigenic variant) and HM175/18f. The genomes of these viruses were found to contain 30 and 33 nucleotide changes from the sequence of HM175/P16 respectively (Table 3) (Lemon et al., 1991). Both viruses shared a common 5' sequence (bases 30-1677), as well as sequence identity in the P2/3 region (bases 3249-5303 and 6462-6781) and 3' terminus (bases 7272-7478). VP3, VP1 and 3C<sup>pro</sup> contained different mutations in the two virus clones, with amino acid substitutions at residues 70 of VP3 and 197 and 276 of VP1 of the antigenic variant. These capsid mutations did not affect virion thermal stability. A comparison of the nearly complete genomic

sequences of three clonally isolated cytopathic variants was suggestive of genetic recombination between these viruses during persistent infection, and indicated that mutations in both 5' and 3' nontranslated regions and in the nonstructural proteins 2A, 2B, 2C, 3A, and 3D<sup>pol</sup> may be related to the cytopathic phenotype.

We are currently using cDNA amplified from HM175/18f virus by PCR to replace related sequences within the infectious pHAV/7 clone. These experiments will define which mutations are necessary for the rapid replication phenotype of HAV, and should result in an infectious cDNA clone of a cytopathic, rapidly replicating variant.

#### 10. Molecular Epidemiology and Genetic Diversity of HAV

In collaboration with Dr. Betty Robertson of the Hepatitis Branch, Centers for Disease Control, we have established an extensive data base containing the partial genomic sequences of over 70 HAV epidemiologically diverse HAV strains. We have determined approximately 170 bases of sequence from each virus at the VP1/2A junction, using the AC/PCR method (Jansen et al., 1990). Development of this method was described in detail in the final annual report of our former contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272) and in Report No. 1 of this grant submitted in 1990. This analysis of partial genomic sequences has demonstrated hitherto unsuspected genetic diversity among human HAV isolates (Jansen et al., 1990). These findings are summarized in Figure 8. Results to date support the existence of 5 distinct HAV genotypes (defined arbitrarily as strains differing at more than 15% of bases sequenced), two of which are indigenous to the cynomolgus monkey. A number of human isolates have been identified which are related closely in sequence to the PA21 strain which was isolated from New World owl monkeys held at the Gorgas Memorial Laboratory in Panama by Binn and Lemon at the Walter Reed Army Institute of Research. The current evidence thus suggests that PA21 is representative of genetically distinct human HAV strains (genotype III), and is not in fact a simian agent.

TABLE 3. Nucleotide changes in cytopathic HM175 virus variants.

Base Position	wild type	p16	clone A (HM175/43c)	clone B (HM175/18f)	clone C (HM175/24a)
5' NTR					
117/118	-	-	-	-	U
152	A	G	G	G	G
154/155	-	-	UUGUAAAUUUGAU	UUGUAAAUUUGAU	UUGUAAAUUUGAU
203-204	UU	dd	dd	dd	dd
551	A	-	G	G	G
591	A	-	G	G	G
647	A	-	C	C	C
687	U	G	G	G	G
P1					
964	A	G	G	G	G
987	U	-	-	-	C
1064	A	-	-	-	G
1678	A	-	C	-	C
1741	C	-	-	A	-
1742	G	A	A	A	A
2036	A	-	-	-	G

TABLE 3. (continued).

Base Position	wild type	p16	clone A (HM175/43c)	clone B (HM175/18f)	clone C (HM175/24a)
2646	A	-	-	-	G
2684	C	-	-	U	-
2780	C	-	-	-	U
2797	A	-	G	-	G
2864	U	-	-	-	A
2930	A	-	-	-	G
3018	U	-	-	C	-
3033	A	-	G	-	G
P2					
3248	A	-	C	U	U
3281	A	G	G	G	G
3557	U	-	-	-	G
3711	G	A	C	C	C
3867	U	C	C	C	C
3889	C	U	U	U	U
4049	C	U	-	-	-
4060	C	-	G	G	G
4066	A	-	G	G	G
4185	G	A	A	A	A
4222	U	-	C	C	C
4272	C	-	U	U	U
4369	C	-	U	U	U
4419	U	-	C	C	C
4426	A	C	-	-	-
4607	U	-	C	C	C
4955	A	-	U	U	U
P3					
5010	GAU	-	ddd	ddd	ddd
5172	U	-	G	G	G
5194	G	-	A	A	A
5204	G	A	A	A	A
5255	A	U	U	U	U
5304	A	-	G	-	-
5592	C	-	-	G	G
5757	A	-	G	-	-
6074	U	-	-	-	C
6148	A	G	G	G	G
6216	U	C	C	C	C
6461	A	-	-	G	-
6522	U	A	A	A	A
6619	G	-	A	A	A
6633	U	-	C	C	C
6782	U	-	C	-	-
6813	C	-	-	-	U
6827	U	-	C	-	C
6920	C	-	-	U	-
7226	U	-	C	-	C
7247	U	-	-	C	-
7253	A	-	G	-	-
7271	U	-	C	-	C
7304	U	-	C	C	C
3' NTR					
7429	C	-	U	U	U
7430	A	G	G	G	G
7433	U	-	C	C	C

Boxed areas represent regions in cytopathic virus clones with mutations (from p16 HM175) identical to those in clone B (- = no change from wild-type; d = deletion). Mutations which occurred during the primary adaptation of HM175 to growth in cell culture (i.e., wt to p16) are included in the table. The sequence of the clone C virus is from S. Feinstone, CBER.

## CONCLUSIONS

During the past year, we have isolated a number of HAV neutralization escape mutants that were selected for resistance to the murine monoclonal antibodies 2D2, AE8, AD7 and H7C27. Partial genomic sequencing of these viruses indicated the involvement of residues 3-074, 1-171 and 1-176 in an immunodominant antigenic site (together with previously defined residues at 3-070, 1-102, and 1-114), and provided new information concerning a second functionally independent antigenic site involving residue 1-221. PEPSCAN analysis continues to confirm the highly conformational nature of the immunodominant HAV antigenic site, but is only now being applied to the 1-221 site. Two additional monoclonal antibodies (H29C26 and 1.193) have been identified which have neutralizing activity for all classes of escape mutants thus far isolated. During the next year, we will isolate escape mutants selected for resistance to these antibodies. Sequencing of the P1 region of the RNA from these new mutants should provide additional information concerning the location of a possible third minor antigenic site. While the 1-221 site (and the putative third yet unidentified site) represent only minor components of the polyclonal antibody response to the virus, we will determine whether one of these sites contains a linear determinant capable of functioning as a synthetic peptide immunogen.

We remain encouraged by the results obtained with the poliovirus/HAV chimeras. The neutralizing activity found in a minority of rabbits immunized with the S1/H15 chimera has been shown to be IgG mediated. These studies have resulted in the identification of an accessory HAV neutralization domain located within the N-terminus of VP1, which is most likely exposed to antibody only after the virus capsid undergoes conformational changes following initial attachment to cellular receptors. The presentation of HAV peptide sequences within the B-C loop of Sabin type 1 poliovirus appears to place important conformational constraints upon the peptide that may favorably influence its immunogenicity. This seems to be the case with the chimeras S1/H2 and S1/H15, as peptide constructs similar to the HAV sequences included in these chimeras have generally failed to elicit anti-HAV neutralizing activity. These ongoing serologic results suggest that chimeric picornaviruses may have promise as possible HAV vaccines, although considerable additional work will be required to identify more immunogenic HAV peptide sequences to include in chimeric viruses. The chimeras are useful tools for further dissection of the antigenic structure of HAV.

We have determined the nearly complete genomic sequences of two cytopathic HAV variants, HM175/18f and HM175/43c. Studies now in progress will define which of the many mutations identified in these variants is necessary for the rapid replication/cytopathic phenotype. This information may be of use in engineering virus variants with enhanced growth for use in vaccine production or diagnostic assays. We report continued progress in construction of an infectious cDNA clone derived from a virulent HAV variant. A lethal, frame-shifting mutation in the pHAV16 clone has been identified and corrected. We anticipate that these efforts will result in an infectious clone from which virulent HAV may be recovered. Such a clone will allow the exploration of new approaches to attenuation of HAV, including the manipulation of the 5' NTR. Our analysis of viruses containing chimeric 5' NTRs derived in part from virulent and attenuated HAVs has indicated an

important role for the 5' NTR in controlling replication in cell culture (Day and Lemon, 1990). We have made substantial progress in identifying the secondary structure and translational control elements of the HAV 5' NTR (Brown et al., submitted for publication). We have shown that it is closely related in structure to the 5' NTR of the murine coronaviruses, and quite distinct from the structure of the poliovirus 5' NTR. We continue to suspect that viable 5' NTR deletion mutants of HAV will be significantly attenuated in primates.

Sequence data generated from over 70 HAV strains using the AC/PCR approach will be useful in analysis of HAV strains isolated during future HAV vaccine trials and in future disease surveillance activities.

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APPENDIX

		Asp 3-070	Gln 3-074	Ser 1-102	Val 1-171	Ala 1-176	Asp 1-221
K24F2	S30	His					
	3A	Asn					
	22A	Asn					
2D2	34A	Asn					
	27A	Asn					
	28A	Tyr					
	7A		Arg				
Cp	43c	Ala					
Spont	L30M	Asn					
B5B3	S32			Leu			
AD2	12A					Asp	
	20D					Asp	
	4C					Glu	
	11c				Glu		
AE8	35M				Glu		
	44K				Glu		
H7C27	15C						Glu
	20B						Met
	33M						Asn
	24B						Asn

Figure 1. HAV neutralization escape mutants, showing the selecting monoclonal antibody and mutant designation (left-hand column), and location and nature of amino acid substitution. Wild-type Hm175 sequence at each residue is shown at the top. CP = cytopathic strain; SP = spontaneous mutant (selected from double antibody radioimmunofocus assay without antibody pressure). By convention, HAV capsid protein amino acid residues are designated by four-digit numbers, the first of which indicates the specific capsid protein, followed by three digits indicating residue number.

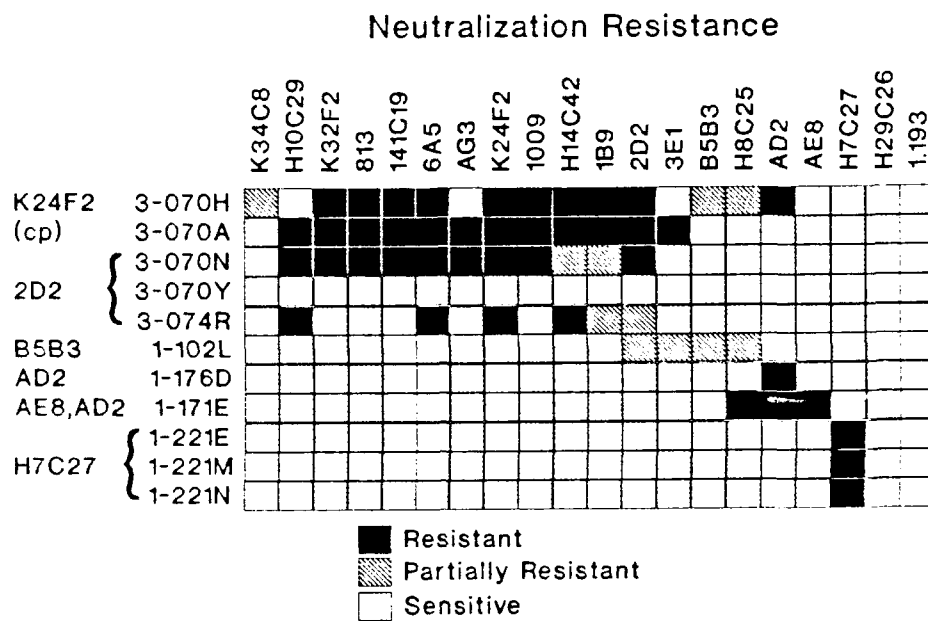
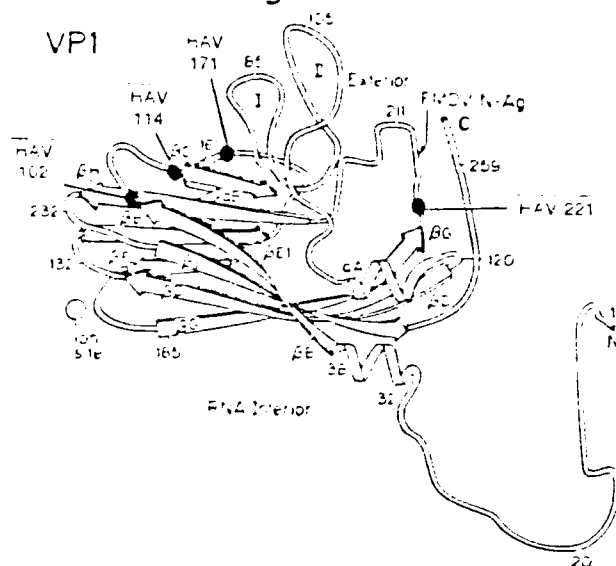
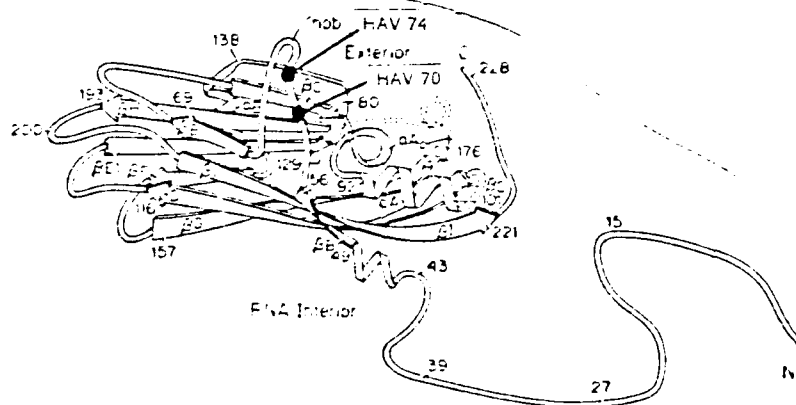


Figure 2. Cross-resistance among HAV neutralization escape mutants. Blank squares represent neutralization tests that are in progress or have not yet been done.

## VP1



VP3



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Chimera	Insert Sequence	HAV Residues	Length
H15	VDN STEQNVDPDPQVG KLF	1013-1024	12
H1	VDN EQNVDP DKLF	1015-1020	6
H11	VD KDLKGKANRGKMD KLF	1029-1041	13
H14	VD ELKPGESRHTSD KLF	1070-1081	11
H10	VD TFNSNNKEY KLF	1099-1107	9
H20	VD TFNSNNKEYTFPITLSSTSNPPHG KLF	1099-1122	24
H2	VDN SNNKEYT DKLF	1101-1108	8
H19	VD ITLSSTSNPPHGL KLF	1111-1123	13
H3	VDN ATDVDG KDKLF	1150-1155	6
H18	VD VDTPWVEKESALS KLF	1166-1178	13
H5	VDN TRRTGN KDKLF	1192-1197	6
H6	VDN GLGDKTDS KLF	1217-1224	8
H12	VD DPRSEEDKRFE KLF	1289-1300	11
H13	VD VDKPGSKKT KLF	2047-2055	9
H17	VD QFPFNASDSVGQ KLF	3063-3074	12
H16	VD ASDSVGQIKVI KLF	3067-3079	11

Figure 4. Poliovirus/hepatitis A virus chimeras that have been studied or are under study for HAV antigenicity. The insert sequence is from HM175 strain HAV, and has been placed in the antigenic VP1 site 1 loop of the attenuated Sabin poliovirus type 1.

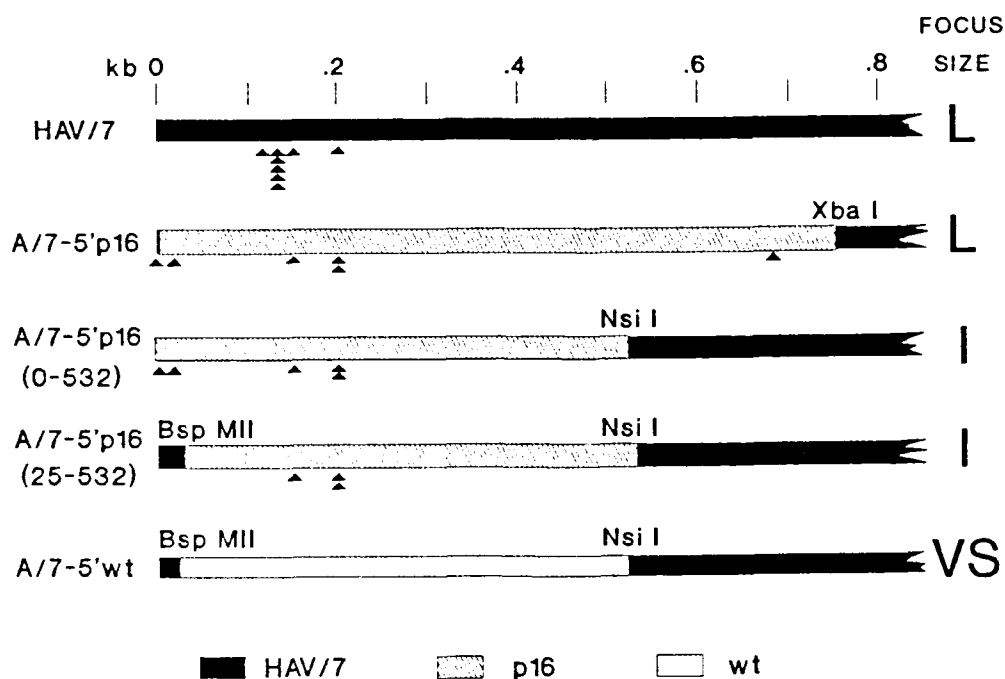


Figure 5. 5' NTR composition of viable HAV chimeras rescued from plasmid DNAs constructed from the pHAV/7 parent. At the right is shown the radioimmunofocus phenotype of the rescued virus in BS-C-1 cells, assessed 14 days after inoculation (L = large, I = intermediate, VS = very small). The arrows indicate the location of point mutations from the wild-type HM175 sequence.

Figure 6. (OVERLEAF) Proposed secondary structure for wild-type HM175 5'NTR RNA. Boxes delineate the sites of covariant nucleotide substitutions upon which the model is derived. Solid arrows indicate sites of cleavage with double-strand specific ribonuclease ( $V_1$ ), while hollow arrows indicate sites of cleavage with single-strand specific nuclease ( $S_1$ ,  $T_1$ , or  $T_2$ ). Shaded areas indicate potential pseudoknot interactions. Major domains are labelled I through VI. The putative initiator codon (AUG-11) is underlined.

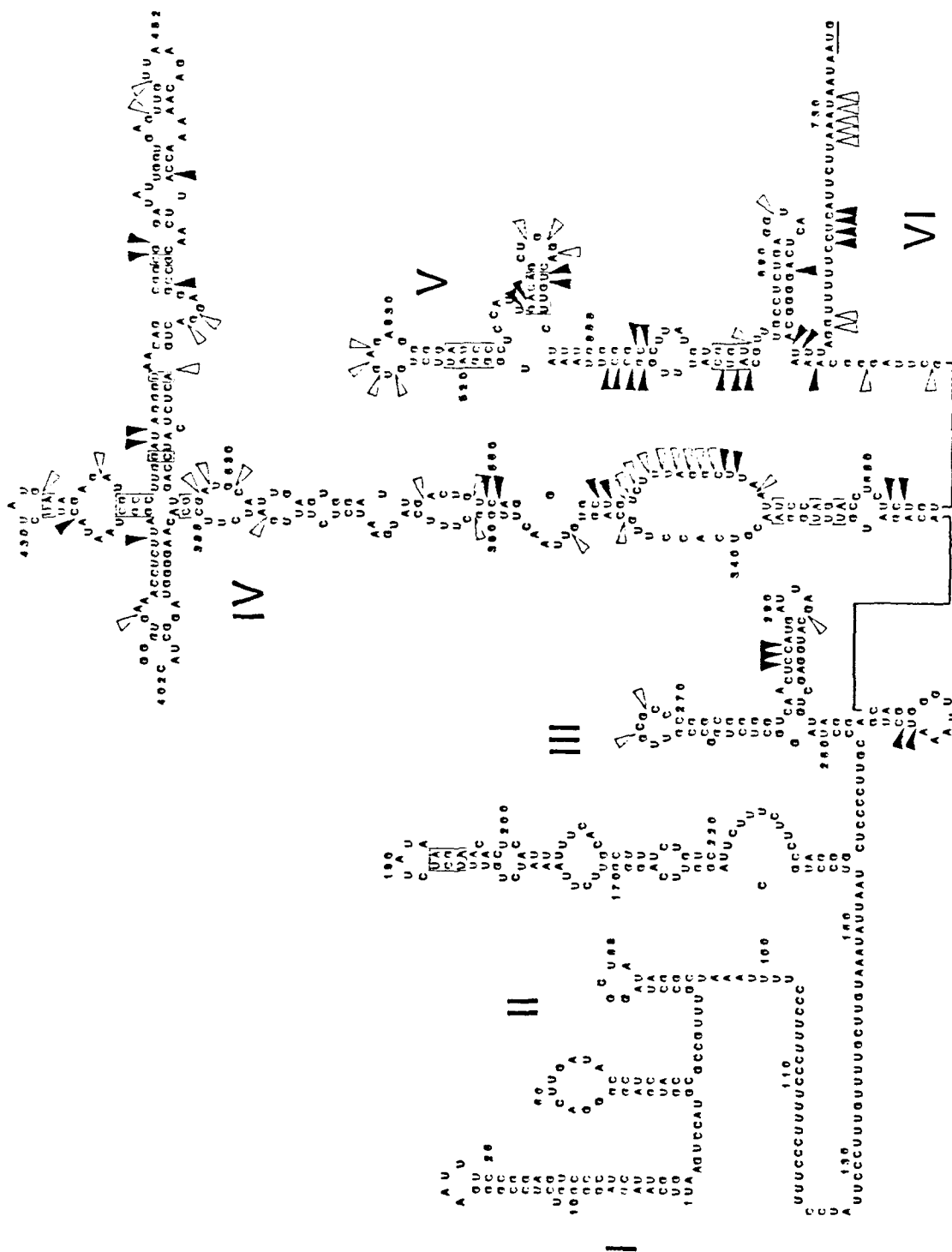


Figure 6. Proposed secondary structure of HAV 5'NTR RNA. Legend on page 29.

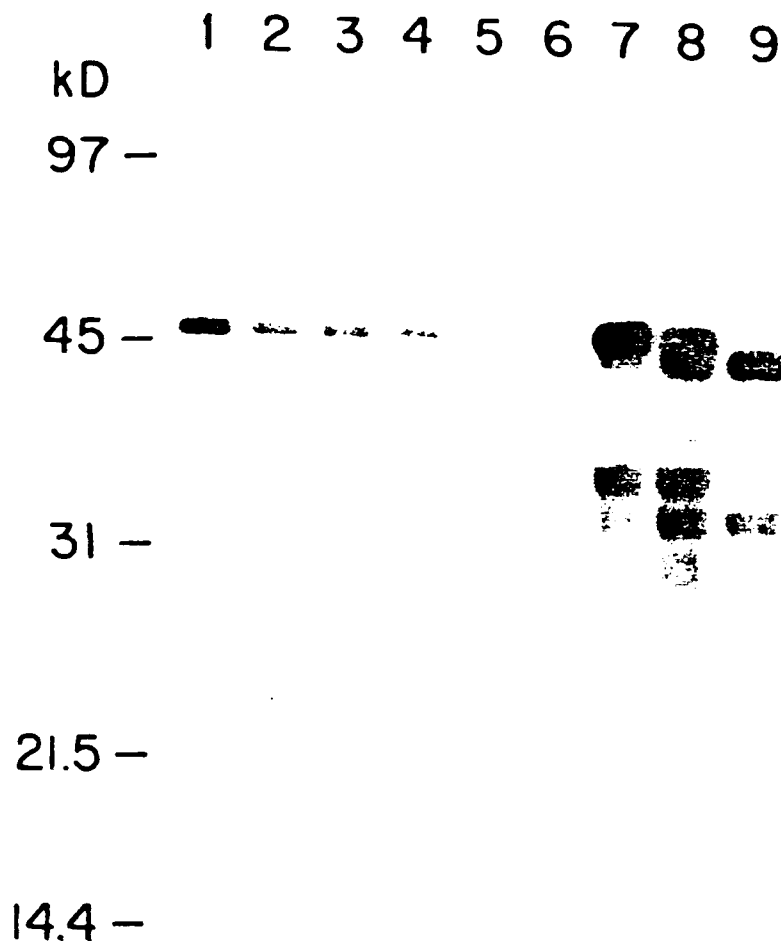


Figure 7. Products of in vitro translation of synthetic HAV 5' NTR RNAs with progressive 5' deletions. Translation products (HAV coding region from base 735-2025) were immunoprecipitated with antibody to recombinant VP0 prior to separation by SDS/PAGE. Lanes: 1, full length 5'NTR of HAV (from plasmid pAccI); 2, p $\Delta$ 46; 3, p $\Delta$ 151; 4, p $\Delta$ 355; 5, p $\Delta$ 447; 6, p $\Delta$ 533; 7, p $\Delta$ 634; 8, p $\Delta$ 740; and 9, p $\Delta$ 745. Plasmid numbers refer to the base position at the 5' terminus of the NTR segment utilized for in vitro translation in rabbit reticulocyte lysates.

Figure 8. (OVERLEAF) Dendrogram constructed by comparative subset averaging of percent nucleotide identity data derived by AC/PCR from HAV strains representing epidemiologically diverse sources. The geographic location and year of collection of each strain is noted. The approximate degree of nucleotide identity between any two strains is represented by the distance from the left of the diagram to the first common node. Roman numerals refer to genotypes, A and B labels to subgenotypes IA, IB, etc.

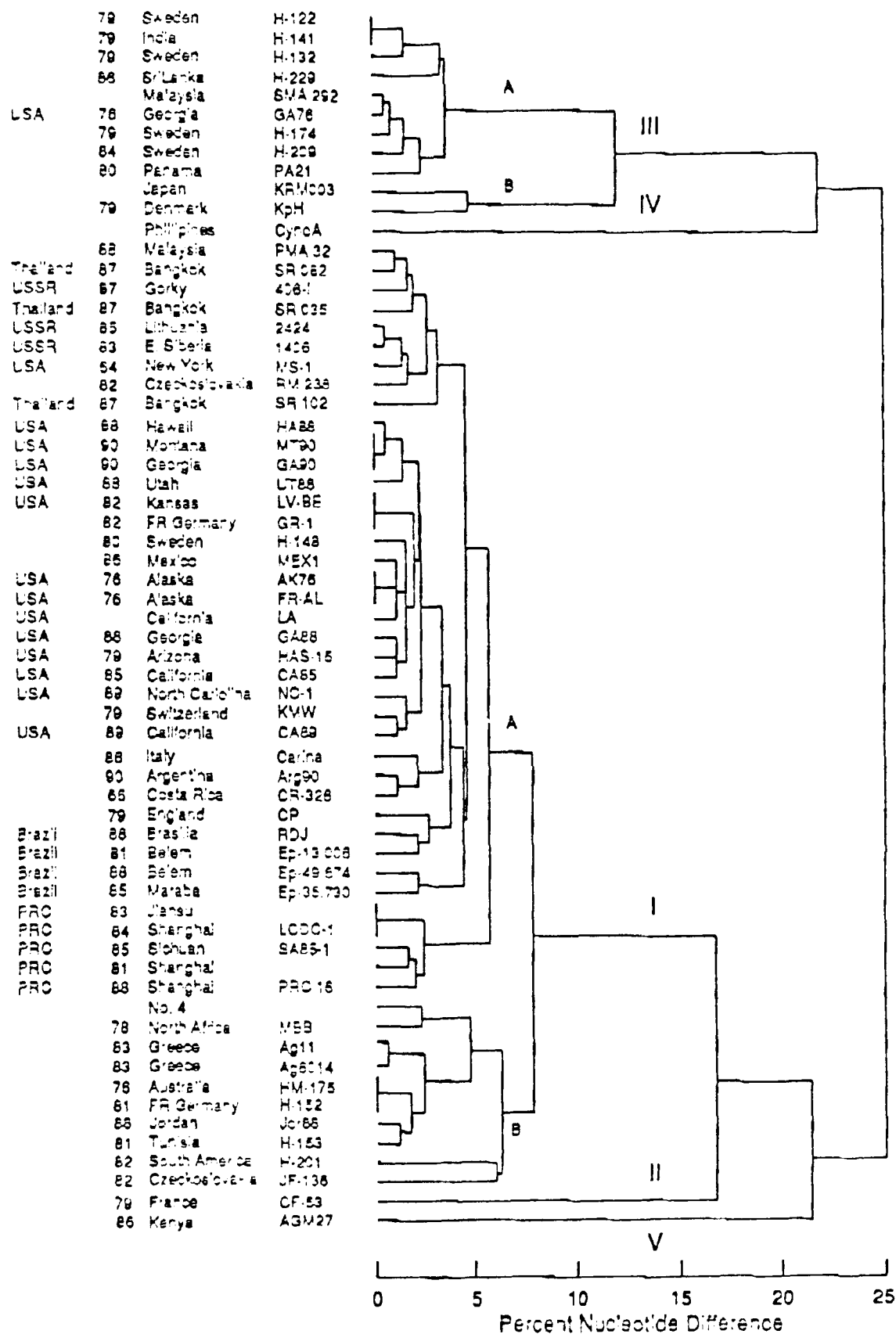


Figure 8. Genetic relatedness among HAV strains. Legend on page 31.